Separation of lactose, lactobionic acid and lactobionolactone by high-performance liquid chromatography☆

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Abstract

Complete separation of the three title compounds has been achieved for the first time on either a β-cyclodextrin- or an aminopropyl-silica gel-bonded-phase HPLC column eluted with acetonitrile–aqueous buffer mixtures. An HPLC system using either of these stationary phases and a refractive index detector directly quantified lactose, lactobionic acid and lactobionolactone at levels between 0.3 and 40 μg of analyte per injection. Simple separations of lactose and lactobionic acid were also readily accomplished on a calcium-form cation-exchange type column eluted with 1.2 m*M* $CaSO_4$ solution. These methods are useful for monitoring the chemical or enzymatic conversion of lactose into the valuable derivatives, lactobionic acid and lactobionolactone.

1. Introduction

Aldonic acids have a broad range of important biological and chemical functions. Aldonic acids such as gluconic acid, for instance, are useful metal chelators because of their ability to form stable complexes with metal ions through coordination complexes involving their carboxyl and hydroxyl groups. Lactobionic acid, 4-O-β-D-galactopyranosyl-D-gluconic acid, has recently gained importance in the pharmaceutical industry by having the ability to solubilize and stabilize drugs such as erythromycin [1] and to preserve the viability of human organs, prior to transplant [2]. Such aldonic acid disaccharides, which can be synthesized by oxidation [3–6] of neutral sugars, establish pH-dependent solution equilibria with their lactone forms.

The separation and quantitative analysis of sugar acids or lactones has been performed by various high-performance liquid chromatographic (HPLC) methods using reversed-phase [7–9], anion-exchange [10–14] and cation-exchange [3,15] columns. HPLC methods have also been developed for the separation of some monosaccharide uronic and aldonic acids from their lactones [16,17] and for separation of uronic acid oligomers [18]. Few methods currently exist, however, for the analysis of glycosyl aldonic acids such as lactobionic acid. One method has been published [3], but it does not permit the direct separation and quantification of lactose, lactobionic acid and lactobionolactone. In our laboratory, we are developing bio-catalytic methods for the oxidation of the surplus dairy by-product, lactose, to produce the valuable deriva-

tives, lactobionic acid and lactobionolactone (4-O-β-D-galactopyranosyl-D-glucono-1,5-lactone). In order to fully characterize the efficiency and selectivity of our bio-catalysts, it was necessary to rapidly quantify amounts of lactose, lactobionic acid and lactobionolactone in reaction mixtures. We now report the first HPLC methods for accomplishing this analysis.

2. Experimental[a]

2.1. Materials

Calcium lactobionate, lactobionic acid and lactose were obtained from Sigma (St. Louis, MO, USA). Lactobionolactone and potassium lactobionate were obtained from Pfanstiehl Labs. (Waukegan, IL, USA). All HPLC solvents were obtained from Baxter (Muskegon, MI, USA) and were purified through 0.45-μm nylon filters. Water used for HPLC analyses was purified using a Milli-Q filtration system obtained from Millipore (Bedford, MA, USA).

2.2. Chromatography

β-Cyclodextrin-bonded-phase columns (Cyclobond I, 250 × 4.6 mm) were purchased from Advanced Separation Technology (Whippany, NJ, USA). Reversed-phase (C_{18}) columns (Econosphere, 250 × 4.6 mm, 5 μm particle size) were purchased from Alltech (Deerfield, IL, USA). The Dynamax-NH_2 aminopropyl-silica gel (APS) columns were purchased from Rainin (Woburn, MA, USA). The HPX-87C column (300 × 7.8 mm) was obtained from Bio-Rad Labs. (Richmond, CA, USA).

The HPLC system used was a Gilson Model 303 dual-pump system equipped with a Model 811 dynamic mixer and a Rheodyne Model 7125 fixed-loop (20-μl) injector. Samples were detected with a Waters Model R-403 differential refractometer and the data were recorded on a Varian 4270 integrator. Samples were also chromatographed with a DuPont Model 8800 pump, equipped with a heated column compartment, a Rheodyne Model 7125 fixed-loop (20-μl) injector, an ERMA ERC-7520 refractive index detector and a Spectra-Physics 4270 integrator.

[a] Reference to brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

3. Results and discussion

3.1. Calcium-form cation-exchange chromatography

Calcium-form cation-exchange columns have been used to separate neutral mono- and disaccharides on both the analytical and the preparative level [19,20]. Hydrogen-form cation-exchange resins have been used for similar separations of uronic and aldonic acids and related lactones [16,19]. There are no reports, however, on the use of calcium-form cation-exchange resins for separation of sugars and sugar acids. When lactose, lactobionolactone and lactobionic acid were chromatographed on the calcium-form cation-exchange column, lactose and lactobionolactone co-eluted, but lactobionic acid was nearly baseline resolved from the other two carbohydrates (Fig. 1). The separations were affected primarily by column temperature and the concentration of calcium ion in the mobile phase. As the temperature was increased from room temperature to an optimal temperature of 85°C, the retention times of all the analytes increased and the peaks became much sharper and symmetrical. The addition of calcium sulfate to the mobile phase increased the retention times of all the compounds, improved the resolution between lactose and lactobionic acid, and regenerated the stationary phase calcium ions that had been removed by complexation with lactobionate. Optimal separations were achieved with a calcium ion concentration of about 1.2 m*M*. The presence of calcium ions did have one disadvantage. Injection of samples containing phosphate and other inorganic anions led to the formation of insoluble calcium salts that precipitated in the HPLC system. Treatment of such samples to

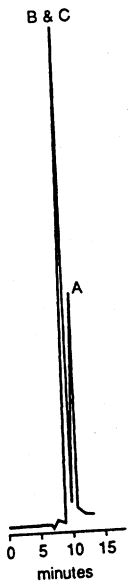

Fig. 1. Chromatogram of (A) lactobionic acid, (B) lactose and (C) lactobionolactone on a Bio-Rad HPX-87C column at 85°C, eluted at 1 ml/min with 1.2 m*M* $CaSO_4$ as the mobile phase. Refractive index detection.

remove inorganic anions, prior to injection, is required.

Since lactose and lactobionolactone co-eluted by this method, the concentration of each of these compounds could not be determined directly. It was possible, however, to saponify the lactobionolactone, using published methods [13,21], prior to HPLC analysis. A quantitative comparison of the resulting chromatogram with that of the unsaponified sample enables one to determine amounts of all three analytes. If one only wants to determine concentrations of lactose and lactobionate (ionized lactobionic acid plus saponified lactobionolactone) this method is simple, rapid, and practical.

3.2. Reversed-phase HPLC

Reversed-phase HPLC has often been used in the separation of carbohydrates [22] and small organic acids [23,24]. Separation of lactose, lactobionic acid and lactobionolactone on a C_{18}-type reversed-phase column produced the optimized separation shown in Fig. 2. Although some resolution of the three species was obtained, lactobionic acid eluted in the void volume of the column along with unretained sample contaminants which made quantification problematic. Lowering the pH of the mobile phase resulted in longer retention times for lactobionic acid, but poorer separation between lactobionic acid and lactose. Reversed-phase columns were, therefore, not considered further for these separations.

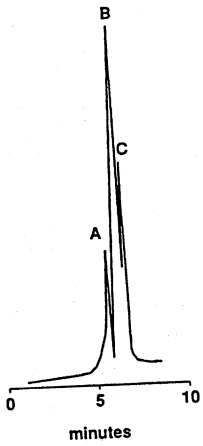

Fig. 2. Econosphere reversed-phase separation of (A) lactobionic acid, (B) lactose and (C) lactobionolactone using distilled water as the mobile phase at room temperature, eluted at 0.5 ml/min. Refractive index detection.

3.3. Aminopropyl-silica gel columns

APS columns are typically used in the normal-phase mode (acetonitrile–water mobile phases) for separation of neutral carbohydrates. Under those conditions, however, acidic carbohydrates bind tightly to the APS phase. Previous workers [25–27] have used APS columns in a weak anion-exchange mode, with buffered mobile phases for separating uronic, aldonic and aldaric acids. Under those conditions, sugar acids are eluted with reasonable analysis times. Since the present separation involved both neutral and acidic carbohydrates, it was necessary for the APS column to function as a weak ion exchanger as well as a normal-phase column. Therefore, a mobile phase of acetonitrile–aqueous buffer was used [25]. The percent of organic modifier and the buffer concentration were varied to determine

the optimized separation conditions. Lactose, lactobionic acid and lactobionolactone were baseline resolved in about 12 min using a acetonitrile–sodium phosphate buffer (50 m*M*, pH 5.0) (60:40) (Fig. 3). Reduction of acetonitrile below the 60% level resulted in poorer resolution between lactose and lactobionolactone. The retention times of the neutral sugars varied little with a change in buffer molarity, but the retention of lactobionate was greatly affected. When buffer molarity was decreased below 50 m*M*, the retention time and peak width of lactobionate increased. When pure water was used as the aqueous component (0 buffer molarity) lactobionate did not elute from the column.

It is important to note that since these three carbohydrates have low solubilities in acetonitrile, the HPLC samples in this case and in those on the β-cyclodextrin column described below were prepared as follows: the sample was first dissolved in buffer and then an equal amount of acetonitrile was added. Failure to follow this order resulted in occasional precipitation of analytes.

3.4. β-Cyclodextrin-bonded-phase column

β-Cyclodextrin-bonded-phase columns are highly selective and have recently been used to separate many types of compounds. Like APS columns, cyclodextrin-bonded phases have the ability to separate analytes by different chromatographic modalities. When a large percentage of organic modifier is used, the column functions in the normal-phase mode [28]. When a large percentage of aqueous component is used, the column functions in the reversed-phase mode [29]. Cyclodextrin-bonded-phase columns have mainly been used for separating chiral compounds [29,30]. To date, the only carbohydrates separated on this stationary phase have been various neutral mono-, di-, tri- and tetrasaccharides, deoxysugars and sugar alcohols [28], and carbohydrate anomers [31].

To achieve separation of lactose, lactobionic acid and lactobionolactone, we used the column in the normal-phase mode. Therefore, various compositions of acetonitrile–water were examined as a suitable mobile phase. Surprisingly, under these conditions, lactobionate was very tightly bound as it had been on the APS column. Hence, a buffered aqueous component was used. The optimized separation of lactose, lactobionic acid and lactobionolactone (Fig. 4) was achieved in approximately 11 min using acetonitrile–sodium phosphate buffer (50 m*M*, pH 5.0) (70:30). A similar separation was achieved in under 10 min using acetonitrile–ammonium acetate buffer (0.2 *M*, pH 5.0) (70:30); however, there was a negative peak that appeared between lactose and lactobionolactone that made quantification of

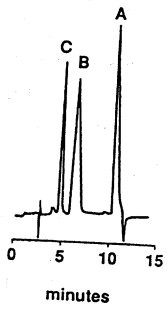

Fig. 3. The separation of (A) lactobionic acid, (B) lactose and (C) lactobionolactone on a Dynamax-NH_2 column using acetonitrile–NaH_2PO_4 buffer (50 m*M*, pH 5.0) (60:40) at 1 ml/min and at room temperature. Refractive index detection.

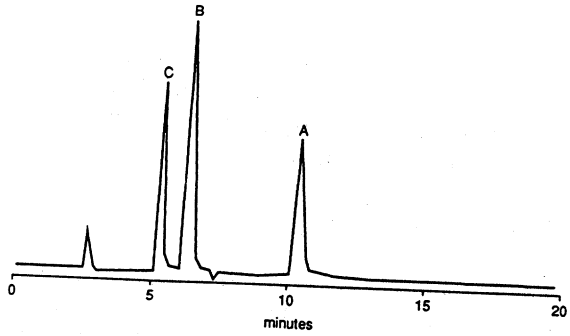

Fig. 4. The separation of (A) lactobionic acid, (B) lactose and (C) lactobionolactone on a Cyclobond I column using acetonitrile–NaH_2PO_4 buffer (50 m*M*, pH 5.0) (70:30) at 1 ml/min and at room temperature. Refractive index detection.

these two carbohydrates difficult. The effect of the molarity of buffer in the mobile phase was examined for this system. As with the APS column, increasing the molarity of buffer led to a decrease in the retention time of lactobionate and, when water was used as the aqueous part of the mobile phase, as mentioned above, lactobionate did not elute from the column. It is important to note that the cyclodextrin column was more stable than the APS column, and over more than two years of use, there was no loss in resolution or capacity. Therefore, the cyclodextrin-bonded-phase column system was most useful and practical for most laboratory applications, such as monitoring the bio-catalytic oxidation of lactose to produce lactobionolactone and lactobionic acid (Fig. 5).

The resolution and the capacity factors for all the different stationary phases used in this work are shown in Table 1. Resolution was calculated as follows: $R_s = \Delta t / t_w$, where Δt is the distance between the two peaks in question and t_w is the average width of the peaks at the baseline. The capacity factor, k', was calculated using the equation $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the peak in question and t_0 is the retention time of a non-retained solute. The data confirm that the best resolution was obtained for all three compounds on the β-cyclodextrin and APS columns.

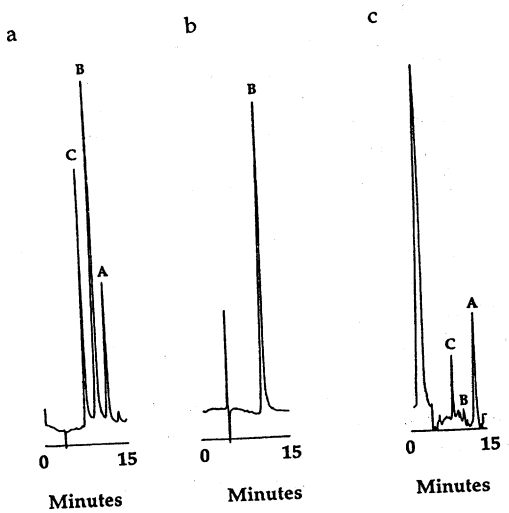

Fig. 5. Bio-catalytic oxidation of lactose monitored by HPLC. (a) Standard lactobionic acid (A), lactose (B) and lactobionolactone (C). (b) Analysis of initial reaction mixture containing lactose. (c) Analysis of reaction mixture after reaction with bio-catalyst. See Fig. 4 for chromatographic system.

Table 1
Capacity factors and resolution values for lactose, lactobionic acid and lactobionolactone obtained for the various HPLC methods

Column[a]	Carbohydrate	k'	R_s
Econosphere	Lactobionic acid	0[b]	
			1.30
	Lactose	0.22	
			0.44
	Lactobionolactone	0.36	
Dynamax-NH_2	Lactobionolactone	0.85	
			1.5
	Lactose	1.32	
			13.1
	Lactobionic acid	2.27	
Cyclobond I	Lactobionolactone	1.12	
			2.6
	Lactose	1.60	
			7.8
	Lactobionic acid	3.28	
HPX-87C	Lactose	0.31	
			0[c]
	Lactobionolactone	0.31	
			1.28
	Lactobionic acid	0.50	

[a] For mobile phases, see Figs. 1–4.
[b] Lactobionic acid eluted in the void volume on this stationary phase.
[c] Lactose and lactobionolactone co-eluted on this stationary phase.

The sensitivity and linearity of detection of lactose, lactobionic acid and lactobionolactone in these HPLC systems were determined. The calcium-form cation-exchange column, APS column and the β-cyclodextrin-bonded-phase column showed linear detector responses from 0.015 mg/ml to at least 2 mg/ml of analyte. With a 20-μl injector loop, this corresponds to a linear range of 0.3 to 40 μg of analyte per injection.

Table 2
Summary of advantages and disadvantages of HPLC methods developed for separation of lactose, lactobionic acid and lactobionolactone

Column	Advantage	Disadvantage
HPX-87C	Simple mobile phase, short analysis time	Lactose and lactobionolactone co-elute; inorganic anions may precipitate as calcium salts
Econosphere	Simple mobile phase, short analysis time	Lactobionic acid elutes in the void volume
Dynamax-NH_2	Baseline resolution of all three compounds in under 12 min	Relatively low solubility of compounds in the mobile phase; stationary phase less stable than others in table
Cyclobond I	Rapid baseline resolution of all three compounds; stable stationary phase	Relatively low solubility of compounds in the mobile phase

4. Conclusions

The relative advantages and disadvantages of each stationary phase for the separation of lactobionic acid, lactose and lactobionolactone are shown in Table 2. For the separation of lactose from lactobionic acid only, use of the HPX-87C column is recommended. This system uses a simple, aqueous mobile phase and a stable stationary phase. If samples also contain lactobionolactone, levels of all three analytes can be determined by quantitative analysis of chromatograms of the samples before and after lactone saponification. Use of the APS or β-cyclodextrin-bonded silica gels will allow the direct analysis of all three compounds. This is the first time a β-cyclodextrin-bonded stationary phase has been applied to separations of sugar acids. Because of its exceptional durability and selectivity, it is highly recommended for this application.

5. References

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